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(54) Title: FRUITS OF THE GENUS CAPSICUM WITH IMPROVED TASTE AND ENHANCED NUTRITIONAL VALUE

(57) Abstract: The present invention relates to a method for obtaining fruits of plants of the genus Capsicum with improved taste and enhanced nutritional value and especially to fruits with increased sucrose and/ or ascorbic acid content as compared to plants of a similar type. The method involves manipulation of the CL and the Y loci of plants of the genus Capsicum

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FRUITS OF THE GENUS CAPSICUM WITH IMPROVED TASTE AND ENHANCED NUTRITIONAL VALUE

The present invention relates to a method for obtaining fruits of plants of the genus Capsicum with improved taste and enhanced nutritional value and especially to fruits with increased sucrose and/or ascorbic acid content as compared to plants of a similar type. The invention also relates to fruits, plants, plant parts and seeds of the genus Capsicum obtainable by said method and to the use of the fruits and especially the use of the fruits for the preparation of food products.

Fruits of plants of the genus Capsicum, like sweet peppers (paprika's) and hot peppers, hereafter both species are referred to as peppers, are available in a wide variety of different colors like red, yellow, brown, and orange for fully matured fruits and green, white, lilac, and purple for non-mature "unripe" fruits. In general, any random non-mature color can develop in any random mature color.

The color of the fruits is a result of a mixture of different color components in the fruit. The color component green is provided by the presence of chloroplasts containing an abundant amount of green chlorophyl. The color component red and yellow are provided by chromoplasts filled with red and yellow carotenoids, respectively. Examples of such carotenoids are capsanthin and capsorubin (red) and violaxanthin and zeaxanthin (yellow). The different possible colors of the mature and non-mature fruits are usually a combination of different ratios between the red, green and yellow color components.

In literature, at least two loci were described to be involved in the color development of the fruits of the genus Capsicum designated the Y and CL loci.

The locus Y controls the development of a red color component in the fruits. Plants comprising the dominant Y allele have fruits with a red color component and plants comprising two recessive y alleles lack a red color component. In detail, plants of the genotype Y/Y, Y/y and y/Y have the phenotypical fruit trait red color component and plants of the genotype y/y do not have a red color component.

The Y and y alleles separate in a Mendelian fashion in crosses i.e. independent of other phenotypical traits, indicating the involvement of a single gene.

The difference between the Y allele and the y allele is a deletion, rearrangement or mutation of a region approximately 25 cM away from the RFLP (Restriction Fragment Length Polymorphism) marker CT204. Recently, it was demonstrated in crosses that the Y locus cosegregates with a gene encoding the enzyme capsanthin-capsorubin synthase (CSS). The enzyme capsanthin-capsorubin synthase (CSS)is involved in the synthesis of red carotenoid pigments in the fruits of plants of the genus Capsicum. Since the location of the capsanthin-capsorubin synthase gene is a genomic region, 25 cM away from the RFLP (Restriction Fragment Length Polymorphism) marker CT204, it is generally accepted that the capsanthin-capsorubin synthase (CSS) gene is the gene responsible for the trait Y, observed in classic breeding experiments.

The locus CL is involved in the transformation of chloroplasts to chromoplasts in plants of the genus Capsicum. During maturation of the fruits, the chlorophyl, responsible for the green color of the fruits, is degraded and the synthesis of carotenoids is initiated. The CL allele is dominant and the cl allele is recessive. Plants of the genotype CL/CL, CL/cl and cl/CL all have the phenotype that the chloroplasts are transformed into chromoplasts in

contrast with the genotype cl/cl whereby the chloroplasts are maintained usually resulting in a lasting green color of the matured fruits.

It was shown that this locus also inherited independently in a Mendelian fashion indicating the involvement of a single gene. However, until now, neither the position of the CL locus on a physical genomic map, nor the specific gene involved in this phenotype could be determined.

In most countries, like the United States and Mexico, the most abundant color of harvested fruits of plants of the genus Capsicum like peppers is green. Every year, in the United states approximately 40.000 ha peppers are harvested green and in Mexico 80.000 ha peppers are harvested green. In general, one can state that most peppers are harvested green and there is a worldwide preference for eating and processing green peppers especially for the preparation of food products.

Although most peppers are consumed while they are still non-matured and thus usually green, the taste of these green non-mature peppers is regarded "less" than the taste of mature peppers, like yellow and red peppers. This is due to the lower sugar content in the non-mature green fruits as compared to the mature fruits. Because of the higher sugar content in mature fruits these fruits are regarded as "tasting" better, i.e. more sweet.

It is therefore a goal of the present invention to improve the taste, i.e. sweetness, of the green fruits of plants belonging to the genus Capsicum.

Peppers are an important source of ascorbic acid (vitamin C) in the human diet. Ascorbic acid is essential for the synthesis of collagen, one of the most abundant proteins in the human body. Additionally, ascorbic acid is important for the inactivation of free radicals, which are byproducts

of the oxidation pathways, and thus important for the prevention of a number of diseases amongst which cancer.

Most people regard oranges as the most abundant source of ascorbic acid, and therefore the fruits of choice for the daily intake of this important vitamin.

Peppers comprise per kilogram fresh weight at least three times more ascorbic acid than oranges and are therefore better suited as a daily source of ascorbic acid. However, Peppers are usually consumed as a "taste" providing ingredient in food products and thus usually not used in quantities sufficient for the daily intake of ascorbic acid. Fruits of the genus Capsicum comprising a higher ascorbic acid content would provide an improved source of this vitamin even in lower quantities.

It is therefore a second goal of the present invention to provide fruits of plants of the genus Capsicum with enhanced nutritional value, i.e. increased concentrations of ascorbic acid, compared to other plants of the genus Capsicum.

In the research that lead to the present invention, the inventors surprisingly found that the above-mentioned two loci, Y and CL, which were previously only identified to be involved in color traits, are also responsible for other unexpected phenotypical traits involving the sugar and ascorbic acid content in fruits of plants of the genus Capsicum.

Therefore the above-stated goals of the present invention, improved taste and increased nutritional value, are provided by the method disclosed in claim 1 of the present invention involving the two loci CL and Y.

The method according to claim 1 improves the taste and/or the nutritional value of fruits of a plant belonging to the genus Capsicum, by manipulation of the CL and the Y

loci preferably resulting in a plant of the genus Capsicum, comprising two recessive y alleles and two recessive cl alleles.

A plant comprising the genotype y/y, cl/cl can be obtained in a number of ways such as using parent plants which comprise a y allele and/or a cl allele.

Since in most cases the presence of a y allele can not directly be determined from the phenotype, by for example color determination of the fruits, due to the dominant nature of a Y allele and the involvement of multiple genes in color development, biological analysis methods, like biochemical and molecular biological methods, are preferably used to determine the presence of a y allele. For similar reasons, a recessive trait and multiple genes, the presence of a cl allele is also preferably determined by using these biological analysis methods.

A parent plant comprising a y allele can for example be selected by using RFLP (Restriction Length Polymorphism). In detail, the genomic DNA of plants of the genus Capsicum can be digested with a restriction enzyme, like DraI, and after gel separation of the fragments, the presence of a polymorphism can be detected with for example a probe recognizing the genomic sequence of the capsanthin-capsorubin synthase (CSS) gene.

The presence of a y allele is indicated by a different sized band on for example a Southern blot as compared with the band indicating the presence of a Y allele. Depending on the nature of the polymorphism, like a deletion, a rearrangement, a mutation, or an insertion, the size of the band indicating the presence of the y allele will be larger or smaller in size. It is well within the skills of the person skilled in the art by using the phenotype of the Y

locus and classical breeding methods to determine which band indicates the y allele.

The presence of a y allele can also be detected by PCR using primers annealing to the 5' end and 3' end of the capsanthin-capsorubin synthase (CSS) gene (GenBank accession number X77289). Plants comprising the y allele are identified by a different sized (compared to the amplification product of the Y allele) specific amplification product.

In addition to identification of a parent plant, an inactivation or inhibition of the capsanthin-capsorubin synthase (CSS) gene and thus a plant comprising a y allele can also be achieved using modern biotechnological methods such as RNA silencing, knock-out, knock-in, anti-sense etc.

Since the chromosomal location nor the gene corresponding to the CL locus is known, a parent plant comprising the cl allele can be identified by selection of plants of the genus Capsicum comprising intact chloroplasts or chromoplasts still comprising chlorophyl in the mature fruits. The presence of chloroplast or chromoplasts comprising chlorophyl in mature fruits can be confirmed by using microscopy or staining techniques for chlorophyl or chlorophyl content analysis like for example HPLC analysis.

After selection of (a) parent plant(s) comprising the y allele and the cl allele, a plant with the genotype y/y, cl/cl can easily be obtained by using classical breeding techniques generally known to the person skilled in the art. For example, starting from parent 1 (genotype Y/Y, cl/cl; brown fruits) and parent 2 (genotype y/y, CL/CL; yellow fruits) the offspring (F_1) will be genotype Y/y, CL/cl. A plant with a genotype y/y, cl/cl can be obtained by a selfing of the F_1. Genetics predicts that the F_2 will comprise 1 out of 16 plants of the y/y, cl/cl genotype.

Plants comprising the genotype y/y, cl/cl are green both in the non-mature and in the mature phase of the fruits. Since the green color of peppers is preferred, such plants also provide for commercial reasons an advantage.

In one preferred embodiment of the invention, the recessive Y locus (y/y) is derived from a plant chosen from the group consisting of Capsicum annuum, Capsicum baccatum, Capsicum frutescens, Capsicum chinense, and Capsicum chacoense, preferably Capsicum annuum. These species are the most commonly used breeds and in addition can easily be crossed amongst each other, thus facilitating obtaining a plant with the genotype y/y;cl/cl after selection of the appropriate parent plant(s).

For similar reasons, the recessive CL locus (cl/cl) is derived from a plant chosen from the group consisting of Capsicum annuum, Capsicum baccatum, Capsicum frutescens, Capsicum chinense, and Capsicum chacoense, preferably Capsicum annuum.

The plants according to the present invention are characterized by an enhanced sugar content in the fruits of the plant relative to the fruits of similar type plants of the genus Capsicum. The higher sugar content in the fruits of plants comprising the y/y;cl/cl genotype, especially in the non-mature phase, provides that the "taste" of such fruits is sweeter as compared to the "taste" of similar type fruits comprising another genotype by increasing the sucrose content by at least a factor 1.5.

More specifically, the plants according to the invention are characterized by a sucrose content of the fruits of more than 5, preferably 5.0, 5.3, 5.6, 5.9, 6.2, 6.3, 6.5, 6.9, 7.1, 14.9,20.1, 23.4, 25, 30, 35, 40 thus 5 to 40, more preferably 5.4, 5.6, 5.9, 6.2, 6.3, 6.5, 6.6, 6.9,

7.1, 13.5, 14.4, 15.4, or 16.8 thus 5.4 to 16.8 grams per kilogram fresh weight.

The plants according to the invention, comprising the y/y;cl/cl genotype are also characterized by an enhanced ascorbic acid content in the fruits of the plant relative to similar type fruits, of any color, of other plants of the genus Capsicum. The higher ascorbic acid content of the fruits by at least a factor 1.3 is especially apparent in the matured fruits.

In more detail, the plants according to the present invention are characterized by an ascorbic acid content of the fruits higher than 2.0, preferably 2.0, 2.3, 2.4, 2.5, 3.6, 4.8, 5.0, 6.5, or 7 thus 2.0 to 7, more preferably 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.85 thus 2.1 to 2.85 grams per kilogram fresh weight.

In another preferred embodiment of the present invention the plants are two commercially available breeds designated "Evergreen 7181", "Evergreen 6203".

The plants of the genus Capsicum with green fruits comprising the genotype y/y;cl/cl, provided by the method according to the present invention, posses advantageous characteristics, like taste and nutritional value, compared to other peppers according to the prior art. Thus the present invention also relates to fruits of plants of the genus Capsicum obtainable with the above-described method. It is obvious to also seeds, seedlings and any plant parts comprising the genotype y/y;cl/cl are encompassed by the present invention.

The fruits according to the present invention can be used for a number of applications. The fruits are particularly useful for the preparation of food products like salads, sauces and other processed foods. In general, green, non-mature paprika's are used for these applications and the

use of the fruits according to the present invention will improve the taste and the nutritional value of these food products.

The invention will be further illustrated by the following examples which are not intended to limit the scope of the invention but are only presented to illustrate the invention, whereby reference is made to the following figures.

FIGURES

Figure 1. Plot illustrating the increased sucrose content in plants comprising the genotype y/y;cl/cl as compared to other plants of a similar type.

Figure 2. Plot illustrating the increased ascorbic acid content in plants comprising the genotype y/y; cl/cl as compared to other plants of a similar type.

EXAMPLES

Example 1, analysis of the sucrose content of peppers comprising different Y and CL loci

The genotype of 5 different peppers was determined, designated "Special", "Oblix", "Evergreen 7181", "Fiesta", and "Evergreen 6203". The peppers comprised the following genotypes, Y/Y;CL/CL, Y/Y;CL/CL, y/y;cl/cl, y/y;CL/CL, and y/y;cl/cl respectively. Plants were grown and fruits were harvested. The sucrose content in the fruits was determined by the following method.

Plant material was grounded in liquid nitrogen into a fine powder. 0.25 gram of frozen material was weighed and mixed with 4 ml of ice-cold 5% meta-phosphoric acid

containing 1mM diethylenepenta-acetic acid. After sonication for 15 minutes and filtration over a 0.2 µm Teflon filter, 150 mg fresh weight of frozen powder was extracted with 1.5 ml of water at 85°C for 30 min, centrifuged for 5 min at 20000g, and supernatant was collected. The pellet was once re-extracted using the above-described conditions. Supernatants were pooled and stored frozen for further analysis.

For analysis, the samples were diluted 10, 50 or 100 times before injection (in order to obtain a signal in the linear range of the detector). Quantification was performed by comparison with external standards. Analysis was performed as described in Sevenier et al. 1996 (*Nat. Biotech.*, 1998, **16**: 843-846) with detector settings according to Dionex instructions for carbohydrate (Dionex technical note 21) The obtained results are summarized in table 1

Table 1

SUCROSE (grams per kilogram fresh weight)				
VARIETY	**A**	**B**	**mean**	**SD**
Special	4.4	4.0	4.2	0.3
Oblix	4.3	2.1	3.2	1.6
Evergreen 7181	6.6	5.9	6.3	0.5
Fiesta	3.3	2.1	2.7	0.8
Evergreen 6203	6.2	6.3	6.3	0.1

Table 1 is graphically presented in figure 1.

From table 1 and figure 1 it is clear that peppers comprising the y/y, cl/cl genotype contain a higher sucrose content than peppers comprising another genotype. The

difference in sucrose content can be as high as more than 1.5 fold depending on the variety used, thus providing peppers with improved "taste".

Example 2, analysis of the ascorbic acid content of peppers comprising different Y and CL loci.

The different genotypes described in the above-given example 1 were also tested for their ascorbic acid content. Plants were grown and the fruits were harvested The ascorbic acid content was determined by the following method

Plant material was grounded in liquid nitrogen into a fine powder. 0.25 g of frozen material was weighed and mixed with 4 ml of ice-cold 5% meta-phosphoric acid containing 1mM diethylenepenta-acetic acid. After sonication for 15 minutes and filtration over a 0.2 µm Teflon filter, 10 µl was injected into a Waters Alliance HPLC system equipped with a photodiode array detector (Waters 996). Ascorbic acid was eluted with 50mM potassium phosphate pH 4.4 at 0.5 ml/min using a YMC-Pro C18 150 x 3.9 mm column set at 30°C. A calibration curve was recorded using a standard curve of free ascorbic acid dissolved in the extraction solution. Recovery of ascorbic acid standard added to the tissues just before extraction was more than 95%., while reproducibility of extraction and analyses of tissues was better than 90%. The obtained results are summarized in table 2

Table 2

ASCORBIC ACID (grams per kilogram fresh weight)				
VARIETY	**A**	**B**	**mean**	**SD**
Special	1.56	1.79	1.68	0.16
Oblix	1.70	1.67	1.68	0.02
Evergreen 7181	2.39	2.22	2.31	0.12
Fiesta	1.28	1.32	1.30	0.03
Evergreen 6203	2.26	2.40	2.33	0.12

Table 2 is graphically presented in figure 2.

From table 2 and figure 2 it is clear that peppers comprising the y/y, cl/cl genotype contain a higher ascorbic acid content than peppers comprising another genotype. The difference in ascorbic acid content can be as high as more than 1.5 fold thus providing peppers with improved nutritional value.

CLAIMS

1. Method for enhancing the "taste" and/ or nutritional value of fruits of a plant of the genus Capsicum by manipulation of the CL and the Y loci.

2. Method according to claim 1, wherein the manipulation results in providing a plant of the genus Capsicum, comprising two recessive y alleles and two recessive cl alleles.

3. Method according to claim 2, wherein the y allele is derived from a plant chosen from the group consisting of Capsicum annuum, Capsicum baccatum, Capsicum frutescens, Capsicum chinense, and Capsicum chacoense, preferably Capsicum annuum.

4. Method according to claim 2, wherein the recessive cl allele is derived from a plant chosen from the group consisting of apsicum annuum, Capsicum baccatum, Capsicum frutescens, Capsicum chinense, and Capsicum chacoense, preferably Capsicum annuum.

5. Method according to claims 1-4, wherein the enhanced nutritional value is characterized by an enhanced sugar content in the fruits of the plant relative to the fruits of a similar type plant of the genus Capsicum.

6. Method according to claim 5, wherein the plant is characterized by a sucrose content which is at least 1.5 times higher than the sucrose content of fruits of a plant of the genus Capsicum of a similar type.

7. Method according to claim 6, wherein the plant of the genus Capsicum is characterized by a sucrose content of the fruits of more than 5, preferably 5 to 40, more preferably 5.4 to 16.8 grams per kilogram fresh weight..

8. Method according to claims 1-7, wherein the enhanced nutritional value is characterized by an enhanced ascorbic acid content in the fruits of the plant relative to the fruits of a similar type plant of the genus Capsicum.

9. Method according to claim 8, wherein the plant is characterized by an ascorbic acid content which is at least 1.3 times higher than the ascorbic acid content in fruits of a plant of the genus Capsicum of a similar type.

10. Method according to claim 9, wherein the plant of the genus Capsicum is characterized by an ascorbic acid content of the fruits of more than 2, preferably 2 to 7, more preferably 2.1 to 2.85 grams per kilogram fresh weight..

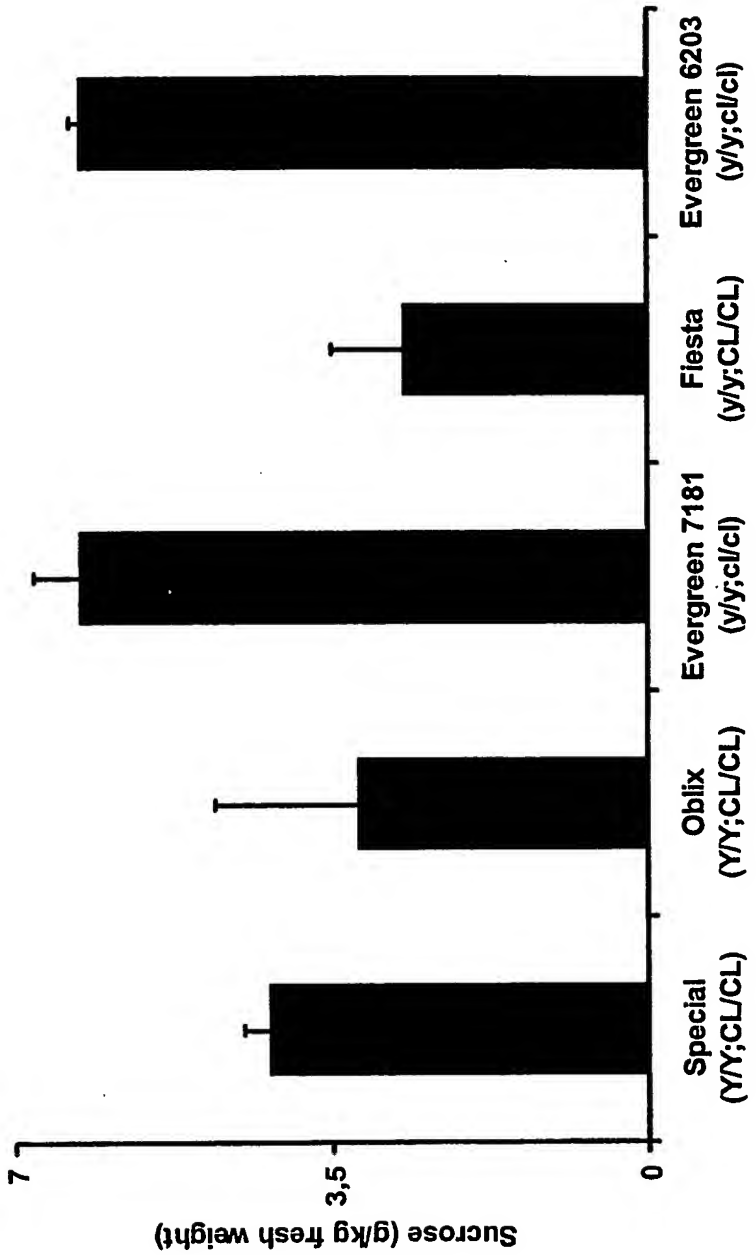
11. Method according to claims 1-10, wherein the plant is "Evergreen 7181", "Evergreen 6203".

12. Plant, fruit, seed, seedling or plant parts of the genus Capsicum obtainable by the method according to any of the claims 1-11.

13. Use of the plant of the genus Capsicum obtainable by the method according to any of the claims 1-11.

14 Use according to claim 13 for the preparation of food products.

Fig. 1



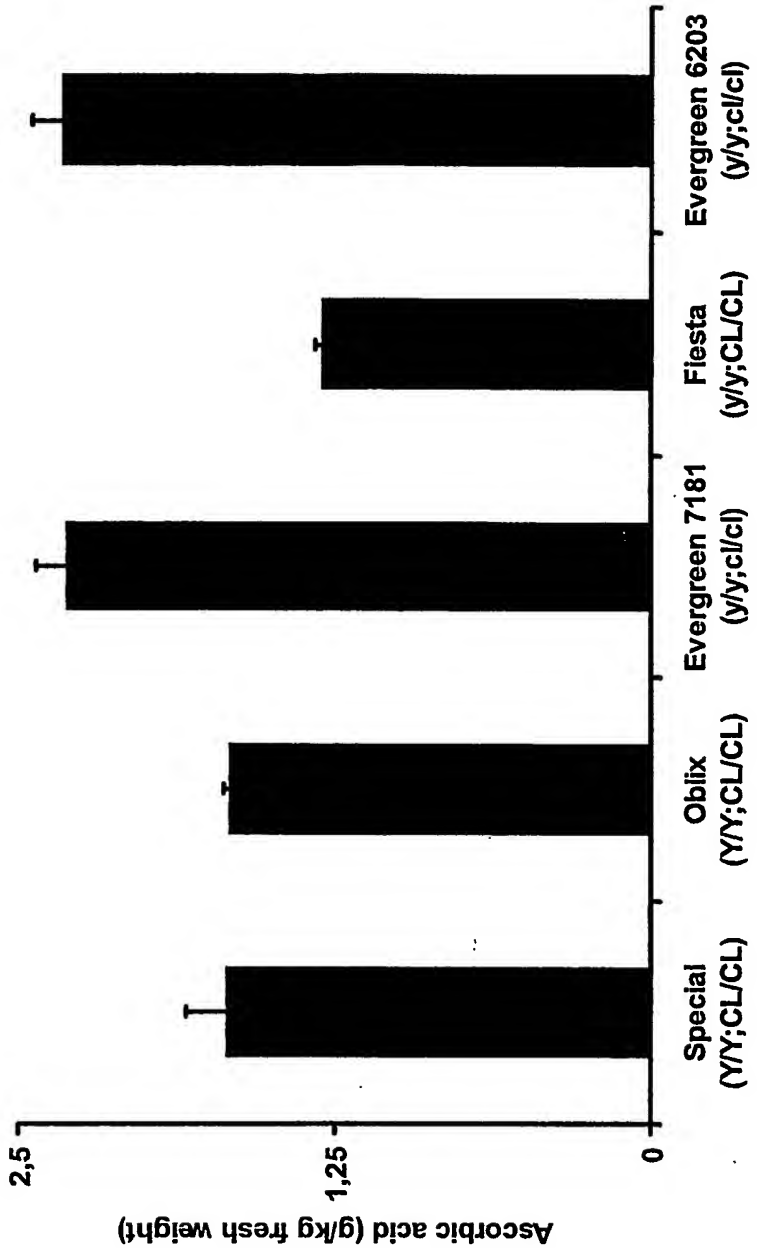


Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/03987

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01H5/00 A01H5/08 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OREN-SHAMIR MICHAL ET AL: "Occurrence of the chromoplast protein ChrA correlates with a fruit-color gene in Capsicum annuum" PLANT MOLECULAR BIOLOGY, vol. 21, no. 3, 1993, pages 549-554, XP009023534 ISSN: 0167-4412 table 1 ---	12
A	DATABASE WPI Section Ch, Week 20327 Derwent Publications Ltd., London, GB; Class A01,Page 001, AN 2003-270523 XP002266414 & HU 0 102 964 A (ANTAL ES TARSA BT), 28 January 2003 (2003-01-28) abstract ---	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 January 2004	22/01/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/EP 03/03987

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 01 62075 A (NASH ALLAN ;DNA PLANT TECHN CORP (US)) 30 August 2001 (2001-08-30)	
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna[redacted]al Application No
PCT/EP 03/03987

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